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TRANSFORMATION AND TRANSFECTION
IN MYCOBACTERIUM

by

Louis A. DeSalle, III

A Thesis Submitted to the Faculty of the Graduate School of
Loyola University (Chicago) in Partial Fulfillment of
the Requirement for the Degree of
Master of Science

March

1971

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INTRODUCTION

Transformation, "the heritable modification of bacterial properties of a bacterial strain by DNA extracted from heterologous bacterial cells," was not known until 1944; however, the first observations related to this mechanism were made in 1928. In 1928, Griffith (28) reported that heat-killed encapsulated pneumococci injected subcutaneously into mice together with living, non-encapsulated pneumococci changed the latter to encapsulated organisms which could give rise to fatal infection of the animal. This important discovery of Griffith was followed up by Dawson and Sia (19), who reproduced the same results under in vitro conditions, and by Alloway (4) who achieved similar results by using sterile filtered extracts of heat-killed cells.

The great breakthrough came, however, in 1944 when Avery, MacLeod, and McCarty showed that the purified material capable of inducing transformation to encapsulated organisms had the properties of deoxyribonucleic acid (DNA) (6).

This could be demonstrated by the study of the physical, analytical chemical properties, and the sensitivity to DNase of the transforming principle (DNA).

There have been repeated demonstrations since 1944 that the principle revealed in the classic pneumococcal transformation applied to several other systems as well.

Balassa was able to show transformation in Rhizobium (7-11). The ability to form nodules on alfalfa, an enzyme necessary for the synthesis of cysteine, and resistance to streptomycin have been transferred by biologically active DNA in Rhizobium.

Besides pneumococcus, transformation could be reliably reproduced in Hemophilus influenzae (2) and Neisseria meningitidis (3). Also, transformation could be characterized in Bacillus subtilis (44). In 1958, Spizizen reported the transformability of several enzymes in B. subtilis (44). Schaeffer and others studied the ability of B. subtilis cells to undergo transformation during the process of sporulation (40). Transformation in B. subtilis cells is one of the best systems thus far studied. Studies in this system have contributed significantly towards clarifying many of the steps involved in transformation.

In 1945, Boivin et al. (12,13,14) reported transformation in Escherichia coli. However, after Boivin's death, the phenomenon could not be repeated by his collaborators neither by numerous experienced investigators in the U.S.A.

Other bacterial species in which transformation has been reported but not yet confirmed are: Shigella paradysenteriae (50), Proteus OX19 (21), Salmonella (20,38), Staphylococcus (22) and Mycobacterium (32,48). More promising results have been obtained with Alcaligenes radiobacter, Phytomonas tumefaciens (18,33), and with Brucella (16).

Katunuma's initial report on transformation in Mycobacterium (32) could not be confirmed in repeated attempts. More recently Tsukamura, Hasimoto and Noda (48) claimed that isoniazid and streptomycin resistance could be transferred to sensitive strains of M. avium by isolated DNA. However these results are not generally accepted.

Evidence is presented in this thesis for the successful transfer of D-xylose utilization to recipient M. phlei F89 xyl⁻ cells by the DNA isolated from donor M. phlei SN109 xyl⁺. Whereas only circumstantial

evidence is presented in this thesis for the successful transfer of streptomycin resistance using DNA extracted from M. smegmatis str-r to competent str-s recipient strains of M. smegmatis. A more detailed investigation of competence in Mycobacterium is presented in the section on transfection in the second part of this thesis.

My interest in transfection stems from the generally held view that transfection by bacteriophage DNA can be considered a simplified model for bacterial transformation.

The purpose of this study, therefore, was to explore the conditions for the uptake of biologically active phage DNA by competent cell populations and to extend the findings for the establishment of a transformation system in mycobacteria.

Transfection in B. subtilis was first reported by Romig (39) using DNA extracted from a phage, SP3, which could infect transformable B. subtilis. Takagi and Ikeda (46) extracted DNA from purified transducing phages and were able to assay the biological activity of bacterial DNA contained in these phages by means of transfection.

Kaiser and Hogness (31) used the DNA of defective bacteriophage λ which can transduce the gal region of E. coli K12 to demonstrate transformation. In this case, however, a "helper" bacteriophage was necessary since the DNA acting alone could not transform E. coli gal⁻ cells.

Harm and Rupert (29) studied transfection of the Rd strain of H. influenzae, using DNA extracted from a clear mutant of a temperate phage. In this system the infectious DNA was sensitive to deoxyribonuclease (DNase) and transfection displayed a linear dependence on the concentration of added DNA.

Földes and Trautner (23) using DNA extracted from bacteriophage SP50 were able to infect B. subtilis cells which had developed competence for bacterial transformation. The kinetics of transfection plotted as a function of DNA concentration was non-linear.

Infectious DNA has been isolated from bacteriophage SP82 (25), ϕ 25 (37) and SPO-1 (36), all of which showed very similar kinetics of infection. Infectious centers appeared at an exponential rate with increasing concentrations of infectious DNA added to competent B. subtilis cells.

Transfection studies have been extended to Mycobacterium by Tokunaga and Sellers (47). All of the transfection systems reported earlier require a particular competent cell population and the competent cells have also the ability of undergoing transformation by bacterial DNA. Mycobacterium is an exception; in this genus no successful transformation has yet been reported. Transfection in mycobacteria does not appear to have an absolute dependence on the stage of bacterial growth; nevertheless, it has been reported that it reaches an optimum in the early stationary phase in the growth cycle (49).

This thesis includes transfection experiments which have been carried out with M. phlei and M. smegmatis using DNA extracted from mycobacteriophage Bo2h. Transfection could be demonstrated during the stationary phase of growth. The reaction was inhibited by deoxyribonuclease (DNase) and was affected by alterations of the temperature.

MATERIALS AND METHODS

a. Bacterial Strains. Mycobacterium smegmatis SN2, SN10, SN46 and M. phlei SN109 of the Borstel collection and M. phlei F89 obtained from Dr. S. Froman, Olive View, California, were used in this study. Strain SN46 naturally resistant to 50 $\mu\text{g/ml}$ streptomycin and a mutant of SN2 resistant to 100 $\mu\text{g/ml}$ streptomycin (str-r), served as donors in transformation experiments, while SN2 sensitive to 1 $\mu\text{g/ml}$ streptomycin (str-s) served as recipient in transformation as well as host in transfection experiments. SN2 str-r cells were selected by streaking a nutrient agar medium containing 100 $\mu\text{g/ml}$ of streptomycin with SN2 and picking surviving colonies. M. smegmatis SN10 and M. phlei F89 acted as recipients in transfection experiments. M. phlei SN109 xyl⁺ served as donor and M. phlei F89 xyl⁻ as recipient in xylose transfer experiments. All bacterial strains were maintained on Löwenstein-Jensen medium prior to use.

b. Mycobacteriophage. Phage Bo2h originally obtained from lysogenic complexes formed between M. phlei and phage phlei Bo2 (Juhász and Bönicke) (30), was preserved in nutrient broth (Difco) subsequent to its propagation in either M. phlei or M. smegmatis host strains. Dependent on the last propagating host, it is designated Bo2h·F89 or Bo2h·SN2. Phage Bo2 of the Borstel collection was used in xylose transfer experiments.

c. Titration of Phage. The titer of phages used in this study was determined by the plaque count method of Gratia (see Adams) (1). Indicator M. smegmatis SN2 or M. phlei F89 organisms were inoculated into 0.75% melted and cooled agar and then poured on top of nutrient agar base plates. After solidification of the top agar layer, 0.05 ml of ten-fold serial

dilutions of the phage were dropped in duplicates, one sample on each half of a plate. After 5 days incubation at 37°C, the plates were assayed. The titer was determined as the number of individual plaques multiplied by the dilution and extrapolated for 1.0 ml of the undiluted suspension (1).

d. Chemicals and Reagents. Phenol was purchased from Allied Chemical Company, Morristown, New Jersey and used in 89% concentration.

Perchloric acid, sodium citrate, sodium chloride, calcium chloride, diphenylamine and disodium (ethylenediamino) tetraacetate (EDTA) were purchased from J. T. Baker Chemical Company, Phillipsburg, New Jersey; Baker analyzed reagent grade.

Cesium chloride (CsCl) optical grade, deoxyribonuclease (DNase) and ribonuclease (RNase) were obtained from Calbiochem, Los Angeles, California.

Lysozyme from egg white was purchased from Nutritional Biochemical Company, St. Louis, Missouri.

Streptomycin sulfate was purchased from Sigma Chemical Company, St. Louis, Missouri.

Glassbeads, 0.10 to 0.11 millimeters in diameter, were obtained from Quigley-Rochester Inc., Rochester, New York.

Buffers. Tris (hydroxymethyl) aminomethane was purchased from Sigma Chemical Company, St. Louis, Missouri. A 1 M stock solution, adjusted to pH 7.6 with HCl, was used to prepare solutions in this study.

SSC: 0.15M sodium chloride, 0.015M sodium citrate was made up from ten-fold concentrated stock solutions. This solution was routinely used for dissolving and storing of DNA preparations.

e. Media. Löwenstein-Jensen (obtained from Co-Lab. Glenwood, Illinois) medium was used to preserve all bacterial strains prior to their use in transformation and transfection experiments.

In both transformation and transfection experiments, nutrient agar and nutrient broth, purchased from Difco Laboratories, Detroit, Michigan, was used. In xylose transfer experiments, purified agar (Difco) and D-xylose (Pfanstiehl) were used.

f. Growth of Mycobacteria in Liquid Culture. M. smegmatis or M. phlei were grown in nutrient broth medium containing 2% glycerol as follows:

(i.) Preparation of the Inoculum. Cells were scraped from Löwenstein-Jensen plates and maintained in the refrigerator for one week and then suspended in nutrient broth to a final cell density of 0.7 optical density units per ml at 620 nm. This corresponds to approximately 10^8 viable bacteria per ml.

(ii.) Inoculation of Nutrient Medium. 500 ml of nutrient broth containing 2% glycerol was inoculated with 10 ml of the above suspension and incubated with shaking in a one liter screw capped flask at 37°C for 4 days. By this method, 3 to 5 grams of wet-weight bacteria were recovered after centrifuging for 10 minutes at 5,000 rpm and washing the cells 3 times with distilled water.

g. Isolation of Bacterial DNA. Method 1. (i.) Mycobacterial cells which had been previously washed 3 times with distilled water, collected by centrifugation for 10 minutes at 5,000 rpm were suspended in 0.10 M NaCl, 0.01 M Tris buffer pH 7.6 and 0.01 M EDTA to a final concentration of 0.5 gm/ml. An equal weight of glass beads (0.10 - 0.11 mm) was added

and the suspension was cooled in an ice-water bath. The cells were broken in a B. Braun cell disintegrator (Quigley-Rochester Inc., Rochester, New York) operating at 4,000 cycles per second, for 5 to 10 seconds.

(ii.) Unbroken cells and glass beads were removed by centrifugation at 5,000 rpm for 10 minutes and the supernatant fraction was carefully pipetted off. (iii.) An equal volume of phenol saturated with the buffer mentioned above, was added to the supernatant fraction and the content of the tube was gently turned over in an ice-water bath 10 times, after which it was allowed to stand in the ice-water mixture for 10 minutes. The procedure was repeated twice and the aqueous layer removed after centrifugation at 10,000 rpm for 10 minutes. (iv.) DNA was collected by centrifugation after 2 volumes of cold 95% ethanol were added to the supernatant and was dissolved in SSC. (v.) RNase was added to the mixture at a final concentration of 50 $\mu\text{g/ml}$ and allowed to digest any RNA present for one hour at 37°C. (vi.) Following RNase digestion, 3 grams of dry, optically pure cesium chloride was added to 2.345 ml of DNA in SSC and the solution was poured into 5 ml Spinco centrifuge tubes, overlayed with 1.5 ml of mineral oil, and centrifuged at 48,000 rpm for 24 hours in the Model-L2-65 Spinco preparative ultracentrifuge. DNA was collected by piercing the bottom of the tubes and recovering the content(s) in 15 ml glass test tubes. DNA fractions located by monitoring the optical density at 260 nm were pooled, dialyzed against several changes of SSC for 24 hours, removed and precipitated with 2 volumes of cold isopropanol. (vii.) Finally, the purified DNA was collected by centrifugation at 5,000 rpm for 10 minutes, and then it was dissolved in sterile SSC to a final concentration of 100 to 200 $\mu\text{g/ml}$ and stored in the refrigerator until used.

Method 2. Mycobacterial cells which had been washed 3 times with distilled water, collected by centrifugation for 10 minutes at 5,000 rpm, were suspended in 10^{-2} M each of NaCl, EDTA, and Tris buffer at pH 7.6. Glycine was added to 1.5% and the final cell concentration was approximately 0.5 gm/ml. The cell suspension was preincubated at 37°C for either 2 or 16 hours. Lysozyme was added at a final concentration of 100 mg/ml and the incubation was continued for an additional 4 or 24 hours. Bacterial lysis was noticeable by a change in the viscosity of the cell suspension. SDS (sodium dodecyl sulfate) was then added at a final concentration of 1% and the mixture was incubated for 15 minutes more at 37°C. The reaction mixture was centrifuged for 10 minutes at 10,000 rpm and the supernatant fluid was decanted, dialyzed for 2 hours against SSC and carried through the same procedure previously described under Method 1, steps iii. through vii.

Method 3. M. phlei SN109 xyl^{+} was grown in H1B for 14 to 21 days. The culture fluid was filtered by millipore filtration and the filtrate was cooled to 4°C. Two volumes of cold 95% ethanol were added to the filtrate and the DNA was collected by centrifugation at 5,000 rpm for 5 minutes, dissolved in SSC to 100-200 μ g/ml. Alternatively, no ethanol precipitation was employed and the biological activity of the DNA in the culture medium was tested directly. By either procedure, large quantities of DNA could be obtained (1-2 mg/ml of culture medium) and it could be stored in the refrigerator for as long as 6 weeks without any loss in its transforming activity.

h. Isolation of Phage DNA. DNA was isolated from phage Bo2h or Bo2 (SN109 xyl^{+}) by the cold phenol method. Phage suspensions containing

1×10^{10} to 5×10^{11} plaque forming units per ml were obtained from a lawn of lysed bacteria by scraping off the soft agar layer from the Petri plates and suspending it in 10 ml of Difco nutrient broth. Unlysed bacteria and nutrient agar were removed by filtration through millipore filter ($0.45 \mu\text{m}$). DNA was extracted by adding an equal volume of liquid phenol to the phage suspension and inverting the capped tube with its content 10 times in an ice-water bath; the mixture was allowed to stand for 10 minutes in the ice-water mixture and the procedure was repeated 3 more times. Denatured protein was removed by centrifuging at 10,000 rpm for 10 minutes. The aqueous layer was carefully taken with a wide-bore pipette and transferred to a sterile 15 ml glass test tube. Two additional deproteinization steps were employed using chloroform-isoamyl alcohol (24/1, v:v). After removal of the aqueous layer, the latter was dialyzed in order to remove the extracting solvent against excess volumes of SSC for 12 to 24 hours. The content of the dialysis bag was transferred to a sterile glass test tube in which the DNA was precipitated with 2 volumes of cold isopropanol and then collected by centrifugation (10 minutes 10,000 rpm). The DNA was dissolved in either 0.01 M Tris-NaCl buffer at pH 7.6 or SSC and kept in the refrigerator (4°C) until used. Burton's diphenylamine method (17) or U.V. absorption were used to determine the amount of DNA in solution. An extinction of 20 was used and this was taken as equivalent to one milligram per ml of DNA at 260 nm.

1. Estimation of DNA in Solution. The amount of DNA in these preparations was determined by the method of Burton (17). DNA was hydrolyzed in 0.5 M perchloric acid (PCA) at $70-75^{\circ}\text{C}$ for 15 minutes; the hydrolysate was brought to room temperature by placing the tubes in a 20°C

water bath. Two volumes of diphenylamine reagent were added and the tubes were incubated overnight at 37°C (approximately 16 to 20 hours). Calf thymus DNA in gradient amounts was used to standardize the reagent. The DNA preparations could be quantitatively estimated from the standard curve. The amount of DNA determined by this method and DNA determined from its U.V. spectrum was in close agreement.

j. Determination of Buoyant Density by Cesium Chloride Density Gradient Centrifugation. Buoyant densities of bacterial or phage DNA were determined by centrifugation at 44,770 rpm at 25°C as described by Schildkraut, Marmur and Doty (42) in a Model E analytical ultracentrifuge. The DNA of Micrococcus lysodeikticus was used as marker ($\rho = 1.7310$).

k. T_m Determination. DNA was adjusted in a 1/10 diluted solution of SSC to a final concentration of 20 µg/ml, and melting temperature profiles were obtained by using a Cary Model 15 spectrophotometer equipped with a Beckman T_m analyzer. Per cent GC was calculated as described by Marmur and Doty (35).

l. Procedure for the Genetic Transfer of the Xylose Marker. In these experiments M. phlei F89 xyl⁻ served as recipient and either DNA of M. phlei SN109 xyl⁺ or phage Bo2 SN109 xyl⁺ served as donor. Ten ml of HIB containing about 3×10^8 bacteria per ml was incubated for 48 hours at 37°C. Transformation experiments were performed in 15 ml screw-capped test tubes containing transforming DNA, controls contained DNA plus DNase or no DNA. 0.3 ml of the above 48 hour cell suspension was added to the reaction tubes and HIB was added to adjust the final reaction volume to 3 ml. The tubes were incubated at 37°C (or 42°C) without shaking for 2½ hours. DNase was added to stop the reaction(s) and the tube(s) were

incubated for an additional 30 minutes at 37°C. The bacteria were collected on a 0.45 μ m millipore filter washed with an excess volume of 0.85% NaCl and 1% D-xylose under gentle suction. After the washing procedure the filters were transferred to capped glass test tubes and the bacteria were suspended in 10.5 ml of NaCl-xylose solution, then incorporated in 1 ml aliquots in 0.75% purified melted and cooled agar and poured on top of a minimal medium base plate consisting of 1% D-xylose as the sole carbon source, 7 gm/l K_2HPO_4 , 2 gm/l KH_2PO_4 , 0.1 gm/l $Mg\ SO_4 \cdot 7H_2O$, 1 gm/l NH_4SO_4 and 15 gm/l of (Difco) purified agar (24).

m. Procedure for the Genetic Transfer of Streptomycin. Recipient strain SN2 str-s grown on Löwenstein-Jensen plates was harvested and suspended in 5 ml of nutrient broth (containing 1.5 μ moles/ml $CaCl_2$) to give a concentration of approximately 5×10^7 cells per ml (0.3-0.4 optical density units per ml at 620 nm). Donor SN2 str-r or SN46 str-r DNA in the amount ranging from 0.1 to 5.0 μ g/ml was added to this suspension. Following incubation at 37°C for 24 or 48 hours and the addition at the end of this period of 20 μ g/ml DNase, the incubation mixture was kept at 37°C for an additional 30 minutes. After this it was mixed with an equal amount of 1.5% nutrient agar containing 100 μ g/ml of streptomycin and pipetted in 5 ml aliquots on top of a 1.5% nutrient agar bottom layer in petri dishes. The final streptomycin concentration amounted to 50 μ g/ml. Str-r transformants were detected by the ability of the originally streptomycin sensitive recipient organisms to grow in the presence of streptomycin. Control samples had no DNA added or DNase was added at the time of addition of transforming DNA.

n. Procedure for Transfection. (i.) M. phlei F89 and/or M. smegmatis SN2 cells were suspended in 25 ml of nutrient broth containing 1-5 μ g/ml

CaCl₂. DNA at varying concentrations was added, and the mixture was incubated for periods of from 1 to 96 hours. The biological activity of the DNA was tested on growing cells at specified time intervals after the addition of DNase. The appearance of infectious centers was tested by filtering the reaction fluid through millipore filters and collecting the effluent in sterile test tubes. Infectious centers were scored as described in this thesis under "Titration of Phage". (ii.) Alternatively M. phlei or M. smegmatis SN2 cells were grown to reach the stationary phase in 25 ml of nutrient broth; 1 or 3 ml aliquots were then taken and DNA at varying concentrations was added for periods of from 1 to 96 hours. Subsequently, DNase was added at specified time intervals to these "stationary cells" and the appearance of infectious centers was scored on F89 or SN2 indicator strains as outlined previously.

RESULTS

Melting Temperatures (Tms) and Buoyant Densities of Phage and Bacterial Nucleic Acids. Buoyant densities of phage and bacterial nucleic acids are recorded in Table 1. The buoyant density in CsCl of DNA is linearly related to its GC content (45). Table 1 shows this relationship for phage and bacterial DNA preparations used in this study. The densities in CsCl for the bacterial strains used in transformation and transfection experiments were as follows: 1.72568 g/cm⁻³ for SN2, 1.72536 g/cm⁻³ for SN46, 1.7310 g/cm⁻³ for F89 and SN109 and 1.7300 g/cm⁻³ for phage Bo2h. Using Micrococcus lysodeikticus DNA with a buoyant density of 1.7310 g/cm⁻³ (35) as a reference marker DNA, we computed the per cent GC for each DNA preparation from the formula of Schildkraut et al. which is: $\%GC = \frac{\rho - 1.66}{0.098}$ (42). Our values for M. smegmatis are in good agreement with those reported in the literature (34), whereas our values for M. phlei are exactly the same as those reported by Schildkraut et al. (42) but differ by approximately 2% when compared to the values calculated by Mandel et al. (34).

We were not able to obtain satisfactory Tm values for our phage or bacterial DNA preparations in SSC due to the high GC content. In SSC Tm values ranged from 95°C to above 100°C. Technically these values are unreliable because melting profiles were obscured at this high temperature due to the limitation of the instrument. Reproducible results were obtained in 10⁻¹ SSC for donor DNAs used in transformation experiments employing the str-r marker. SN46 DNA melted at 73.8°C while we obtained an average Tm value of 75.1°C for SN2 DNA (Figs. 1 and 2). When these

	ρ g/Cm ⁻³	% GC
<u>M. smegmatis</u>		
SN2	1.72568	67.3
SN46	1.72536	66.3
<u>M. phlei</u>		
F89	1.7310	72.0
* SN109	1.7310	72.0
Phage Bo2h	1.7300	71.0

TABLE 1. % GC content of mycobacteria and mycobacteriophage Bo2h as determined by cesium chloride density gradient centrifugation. These values were obtained consistently in at least three analyses.

* Prepared by either method 1, 2 or 3 (see Bacterial DNA Preparation in Methods Section of this Thesis).

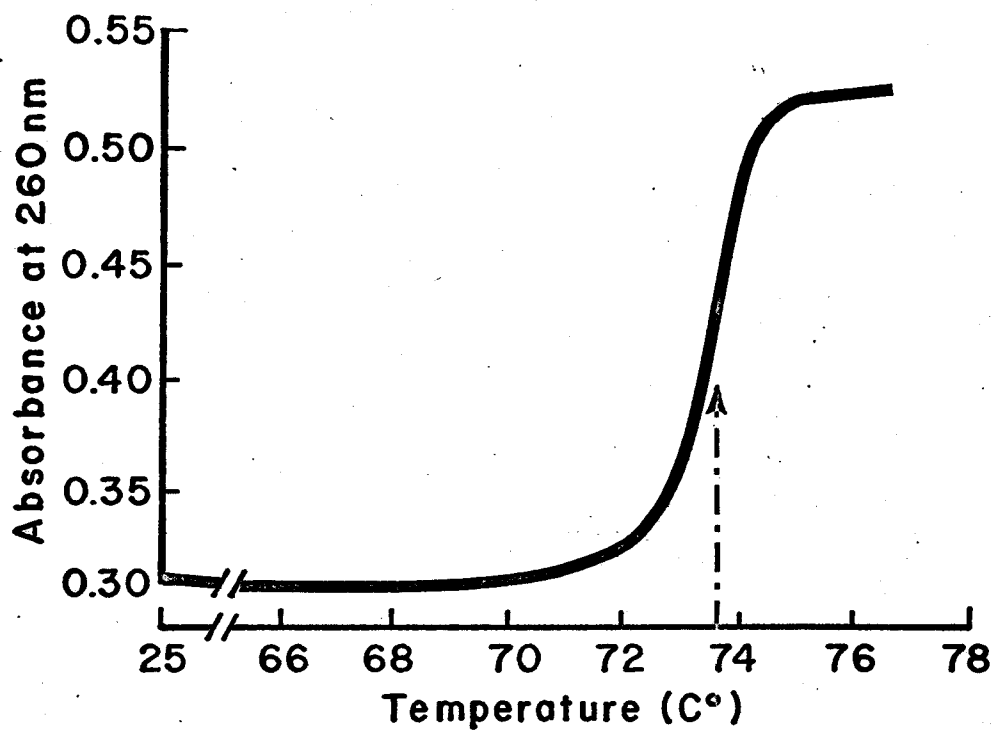


FIGURE 1. T_m profile of SN2 DNA in 10⁻¹ SSC. The final DNA concentration was approximately 20 µg/ml.

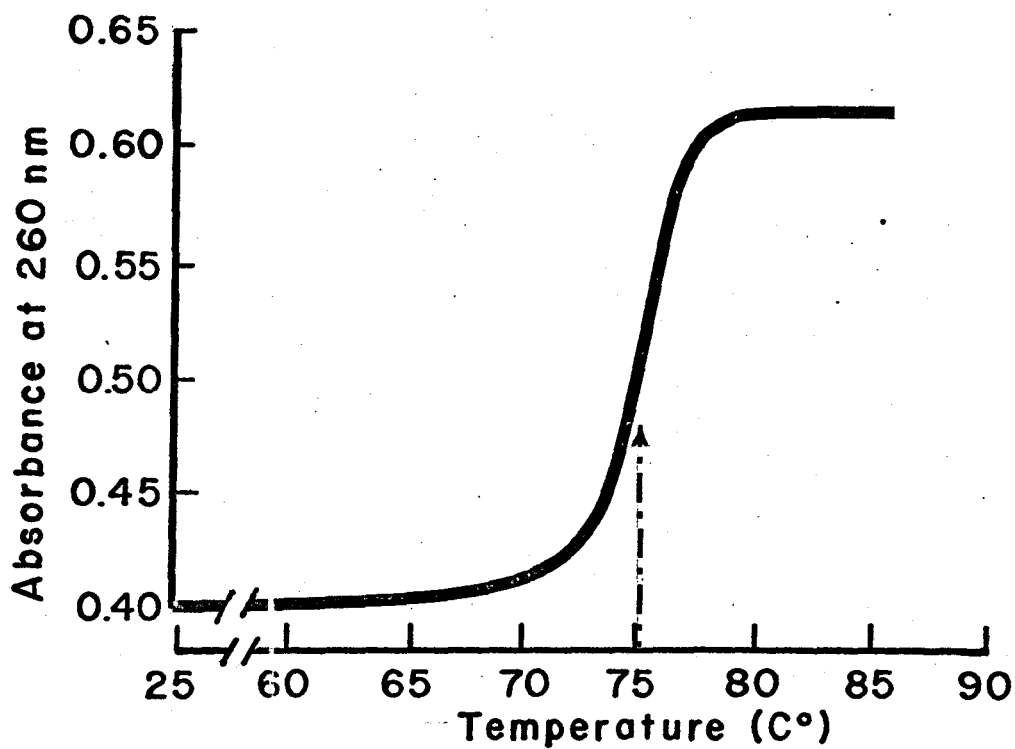


FIGURE 2. T_m profile of SN46 DNA in 10^{-1} SSC. The final DNA concentration was approximately $17 \mu\text{g/ml}$.

values were compared to calf thymus and micrococcal DNA preparations which were melted under the same conditions, we calculated a GC content of 70% for SN2 and 67% for SN46 DNA preparations.

The Genetic Transfer of D-Xylose. In these experiments M. phlei F89 xyl^- as recipient and either DNA of M. phlei SN109 xyl^+ or that of Bo2 SN109 xyl^+ served as donor. Recipient F89 xyl^- was incubated with donor SN109 xyl^+ or Bo2 SN109 xyl^+ in 3 ml of HIB containing 1-5 $\mu\text{moles/ml}$ CaCl_2 for $2\frac{1}{2}$ hours. DNase was added to terminate the reaction and the bacterial population was then plated and tested for its ability to grow in a minimal medium with D-xylose as sole carbon source.

We observed that transformation to xylose utilization occurred when a 48 hour culture of the recipient was used. This roughly corresponds to the stationary phase in the growth cycle of recipient F89 (Fig. 3). This was not an unexpected finding, since transfection experiments reported in the second part of this thesis showed that competence in a population of F89 cells was achieved during the stationary phase of growth. The transfer of D-xylose is summarized in the data of Table 2.

The Genetic Transfer of str-r. Recipient SN2 str-s was cultured with donor SN2 str-r or SN46 str-r in 25 ml of nutrient broth. Samples were removed after 48 hours and DNase was added to terminate the reaction. The total bacterial population was then plated and tested for its ability to grow in the presence of 50 $\mu\text{g/ml}$ of streptomycin. We observed that transformation to streptomycin resistance occurred when a 48 hour culture of the recipient was used. This roughly corresponds to the stationary phase in the growth cycle of recipient SN2 (Fig. 4).

Transformants were scored at approximately one week intervals for a total of 5 weeks. Although we found a 1% difference in GC by buoyant

Experiment Number	μ gDNA/ml		DNase	† Total Number of Transformants/3 ml
	Bacterial	Phage		
1	0.5	--	-	2073
	* 0.5	--	+	240
	0.0	--	-	265
2	2.5	--	-	874
	* 2.5	--	+	22
	† 2.5	--	-	23
3	---	2.0	-	109
	---	* 2.0	+	0
	---	† 2.0	-	0

TABLE 2. Transformation of the xylose marker by the DNA of M. phlei SN109 xyl⁺ and phage DNA Bo2-SN109 xyl⁺ in competent (48 hour stationary culture) M. phlei F89 xyl⁻. The final number of recipient bacterial population was 2×10^{10} in Exp. 1 and 5×10^9 CFU/ml (colony forming units/ml) in Exp. 2.

* DNA plus 20 μ g/ml of DNase was preincubated for 30 minutes before competent F89 xyl⁻ cells were added.

† Experiment done at 42°C.

‡ The final volume was 3 ml and the total number of transformants was obtained from 10 plates. These experiments were done with the DNA prepared by Method 3.

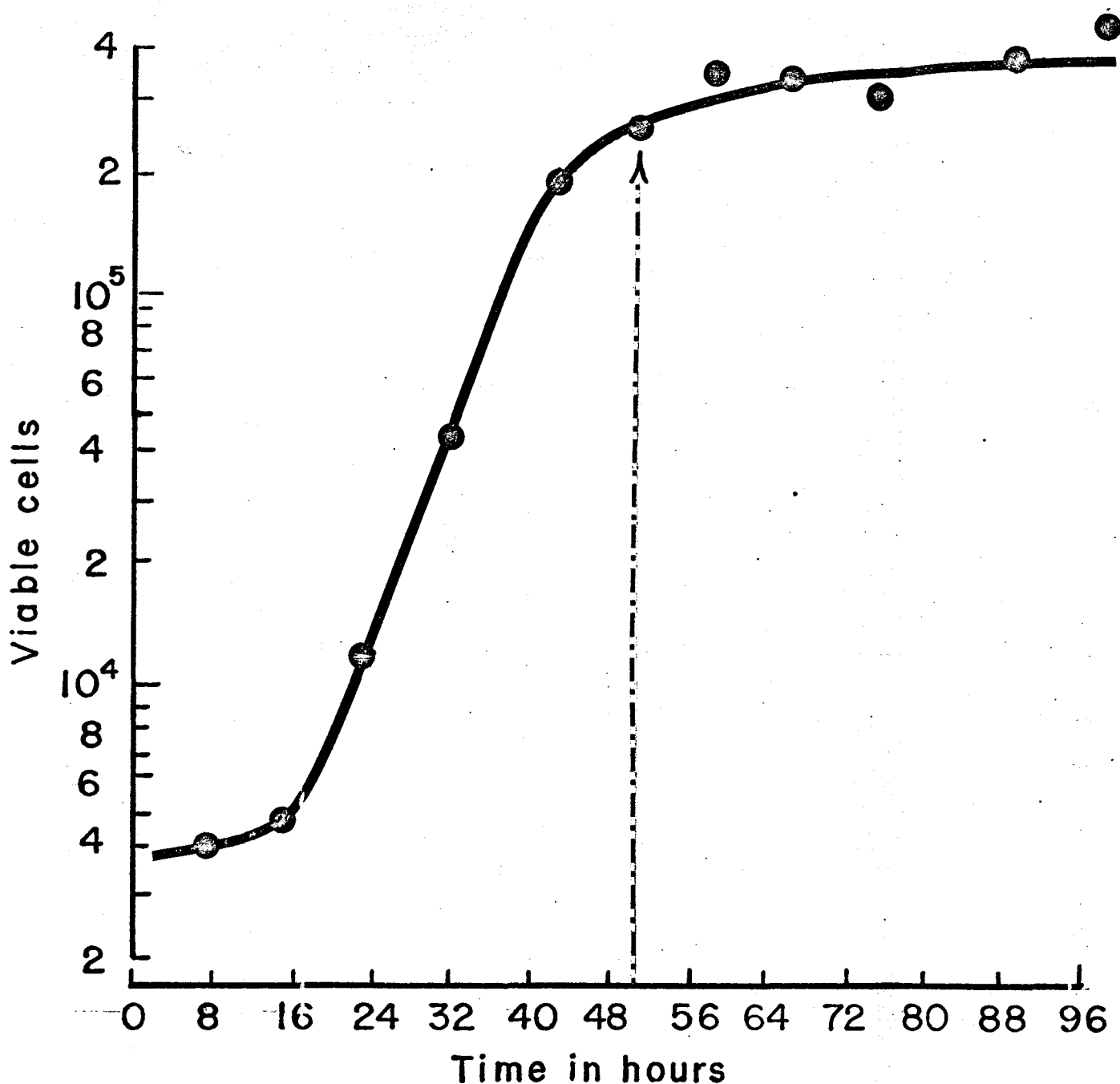


FIGURE 3. Growth curve of *M. phlei*. 100 ml of HIB (heart infusion broth) was inoculated with 3.8×10^3 cells. Incubation was carried out for a total of 96 hours. Aliquots were taken at 8 hour intervals and viable counts were made. The arrow indicates the stage at which competent cells were used in our transfection experiments. (This graph is courtesy of Sheldon Gelbart and James Kraiss.)

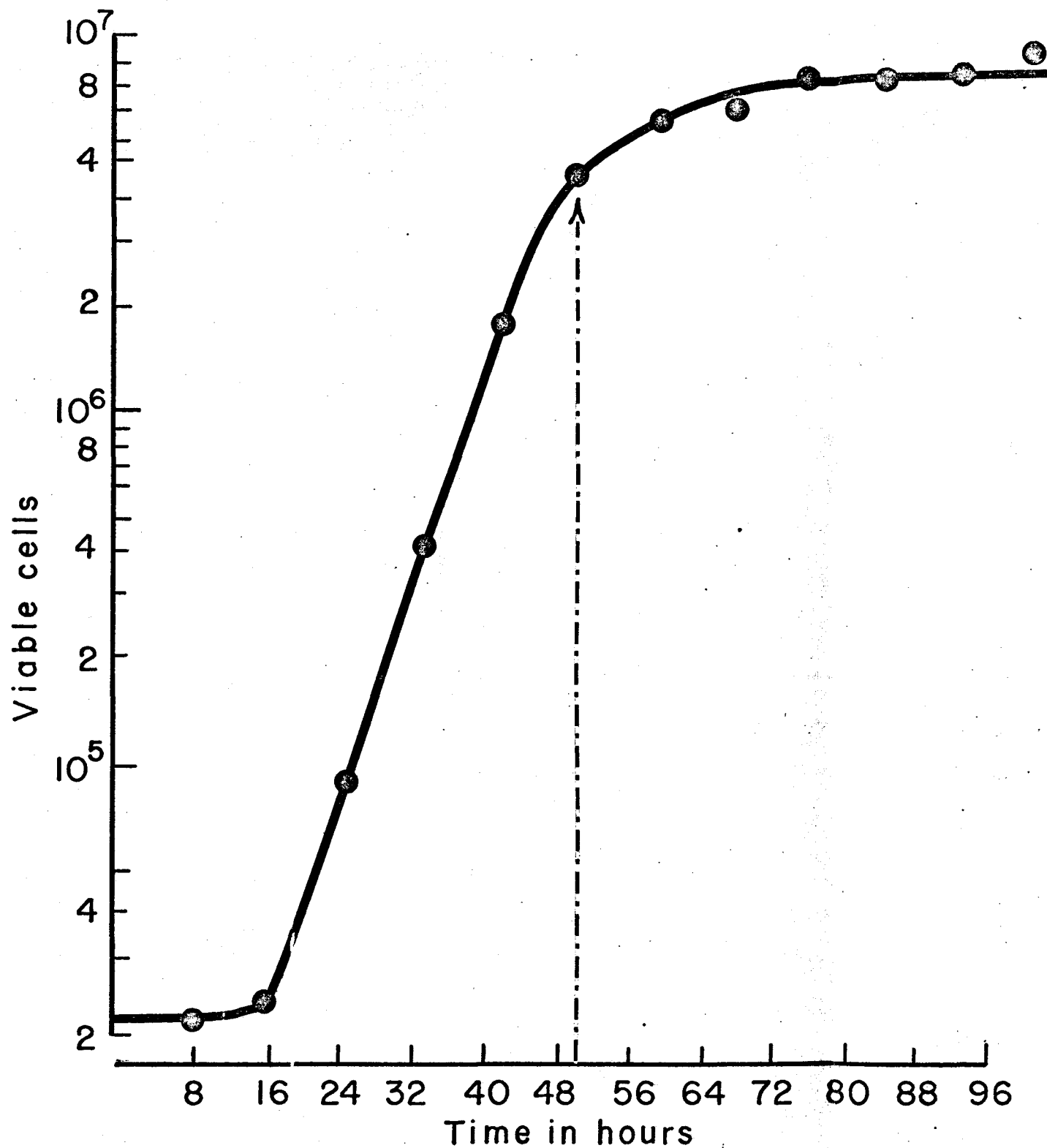


FIGURE 4. Growth curve of *M. smegmatis* SN2. 100 ml of HIB (heart infusion broth) was inoculated with 2.1×10^4 cells. Incubation was carried out for a total of 96 hours. Aliquots were taken at 8 hour intervals and tested for viability. The arrow indicates competent cells used in our transformation and transfection experiments. (This graph is courtesy of Sheldon Gelbart and James Kraiss.)

density measurements between SN2 and SN46 DNA preparations, we consider this difference insignificant in respect to our transformation results since SN46 DNA seemed to act even better on SN2 than homologous SN2 DNA preparations. As it can be noted from the data in Table 3, a slightly higher frequency of transformation was observed when SN46 DNA was used as donor. 2.14 times more colonies were able to grow in the presence of streptomycin after SN2 str-s cells had been treated with SN46 str-r DNA than in control samples which were not exposed to transforming DNA. By contrast, the level of streptomycin resistance acquired by cells exposed to donor SN2 str-r DNA was only 1.72 times greater than that of controls. Furthermore, if the DNA preparations were treated with DNase prior to contact with recipient cells, the number of transformants were similar to those in the controls (indicating spontaneous mutation to str-r — See Table 3). Although the level of streptomycin resistance transferred by donor DNA preparations was not strikingly high, the aforementioned results were reproducible in repeated experiments. It is merely suggested, therefore, that streptomycin resistance may have been transferred by mycobacterial DNA.

Transfection. Prior to their use in transformation experiments, the ability of recipient bacterial strains to take up biologically active phage DNA was tested. The rationale for these experiments was based upon the results of Bott and Wilson (15) who showed that B. subtilis strains, capable of transfection, were also transformable under the same conditions of growth. They showed that the development of competence for transfection parallels that for transformation, and concluded that transfection by bacteriophage DNA provided "a valid assay for transformation".

Reading After	SN46 DNA	SN2 DNA	Control No DNA	SN46 DNA + DNase
14 days	74	56	34	33
23 days	278	231	123	103
31 days	287	238	139	121

TABLE 3. Transformation of a 48 hour culture of M. smegmatis SN2 str-s to streptomycin resistance by the DNA of SN46 str-r and SN2 str-r.

Numbers in columns represent total number of colonies which in a population of approximately 10^9 can grow on a medium containing 50 μ g streptomycin per ml.

In the course of these experiments, competence of the mycobacterial cell population was reached during the stationary phase of growth (shown by arrow(s) in Figs. 3 and 4). Although Seller and Tokunaga reported that no specific stage in the growth cycle was needed for transfection in Mycobacterium, they nevertheless found that early stationary cultures produced optimal results (43).

The data in Table 4 illustrate the infectivity of Bo2h.F89, Bo2h.SN2 and the DNA extracted from these phages when tested on recipient strains of M. phlei F89 and M. smegmatis SN2 and SN10. Mycobacteriophage Bo2h propagated on strain F89 was infectious in addition to M. phlei F89 for M. smegmatis strains SN2 and SN10. DNA extracted from this phage was also infectious for these strains but had, as expected, a lower number of plaque forming units (PFU). The highest number of PFU was obtained with the intact phage whereas it was decreased by approximately 10^{-6} when the DNA was extracted and tested against the same host strains. Upon propagation of Bo2h on SN2, Bo2h.SN2 had a lower number of PFU than Bo2h.F89 for F89 or SN10, but had a higher number of PFU for SN2 than had Bo2h.F89 for the same. DNA extracted from Bo2h.SN2 was never infectious for any of the host strains used in these experiments. Variation of temperature as well as use of the growth stage, which was successful for transfection experiments, lead consistently to negative results with Bo2h.SN2 DNA. Figures 5, 6 and 7 illustrate the results of several transfection experiments using mycobacteriophage Bo2h.F89 DNA. It was in these experiments that a competent population of M. phlei and M. smegmatis cells were detected during the stationary growth phase. It was found that independent of whether cells were scraped from a Löwenstein-Jensen

Host Strain		Bo2h·F89			Bo2h·SN2		
		Intact Phage	DNA	DNA + DNase	Intact Phage	DNA	DNA + DNase
<u>M. phlei</u>	F89	2×10^{11}	1×10^5	0	4×10^6	0	0
<u>M. smegmatis</u>	SN2	7.2×10^8	CL (+)*	0	8×10^9	0	0
<u>M. smegmatis</u>	SN10	3.6×10^7	1.3×10^5	0	1×10^3	0	0

TABLE 4. Numbers in columns represent total number of plaque forming units per ml obtained by allowing the reassembled phage particles to recycle when either intact phages Bo2h·F89 and Bo2h·SN2 or their 2.3 µg/ml DNA were reacted with the indicated bacterial strains at 37°C for 48 hours. Transfection reactions were terminated by the addition of 20 µg/ml DNase.

* CL (+) indicates that no individual plaques were formed but an area of hazy confluent lysis appeared (see text for explanation).

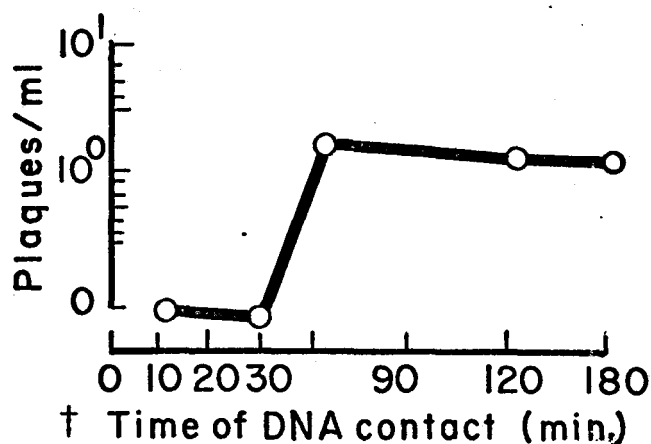


Figure 5. Kinetics of DNA uptake by a competent population (48 hours) of M. phlei strain F89. Approximately $10^8 - 10^9$ cells were incubated at 37°C with Bo2h·F89 bacteriophage DNA (2.3 $\mu\text{g/ml}$) for various time intervals. The reactions were terminated by the addition of DNase (20 $\mu\text{g/ml}$).

† After 180 minutes the number of PFU/ml dropped to zero and infectious centers reappeared only after 48 hours or more of additional incubation (see Fig. 7).

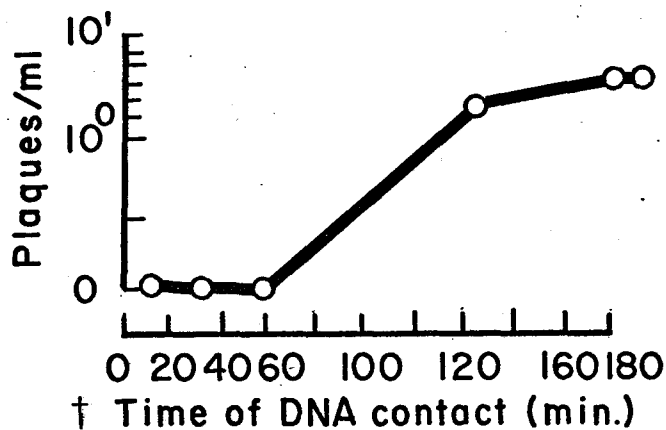


Figure 6. A competent population (48 hours) of M. smegmatis strain SN2 was incubated at 42°C with Bo2h·F89 bacteriophage DNA (2.3 µg/ml).

† After 180 minutes the number of PFU/ml dropped to zero and infectious centers reappeared only after 48 hours or more of additional incubation (see Fig. 7).

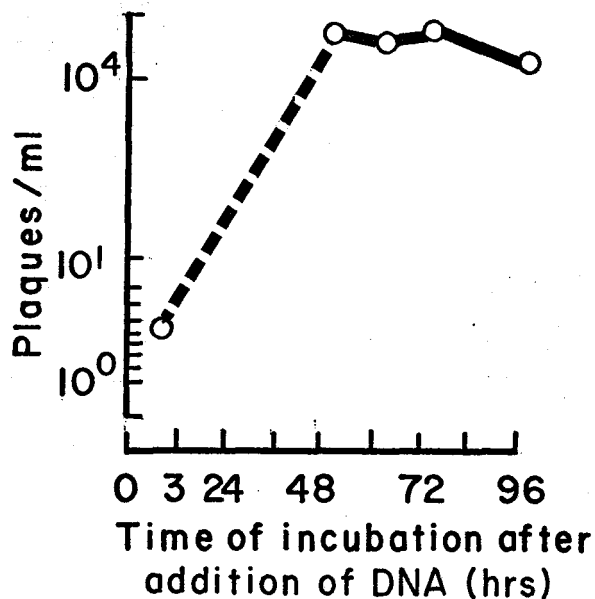


Figure 7. Kinetics of DNA uptake by a competent population (stationary cells) of *M. phlei* strain F89. The cells were incubated at 37°C with Bo2h-F89 bacteriophage DNA (2.3 µg/ml) for various time intervals. The reactions were terminated by the addition of DNase (20 µg/ml).

o-----o In the period between 3 hours and 48 hours no infectious centers were detectable with 48 hour competent bacterial population serving as recipient.

o-----o Infectious centers reappeared after 48 hours incubation. The points represent total number of phage particles obtained upon recycling for 48 hours.

plate, suspended in nutrient medium and then transferred in tubes containing phage DNA at the proper concentrations, or cells were first grown in broth to reach stationary phase then transferred in the proper concentration to fresh nutrient medium containing phage DNA, transfectants appeared. The period of competence was transient, lasting only from 2 to 3 hours, and the number of plaques detectable during this period was generally very low (Figs. 5 and 6). If cells taken from a 48 hour culture were allowed to recycle to the stationary phase in the presence of phage DNA, the number of infectious centers increased markedly (Fig. 7).

The Dependence of Transfection Upon Phage DNA Concentration. In order to relate the number of plaques produced per molecule of infectious DNA, the kinetics of transfection at various DNA concentrations was studied. The data illustrated in Figure 8 show that the relationship between the amount of DNA (added to competent cells) and the number of plaques recovered is not linear. In addition, we observed a very narrow range of DNA concentration in which infectivity appears (2 - 2.3 $\mu\text{g/ml}$). It was found that higher or lower concentrations of phage DNA resulted in loss of recoverable infectious centers.

The Effect of Variation of Temperature on Transfection. When the effect of various temperatures on transfection was studied, it was found that DNA extracted from Bo2h-F89 was infectious for M. phlei F89 and M. smegmatis SN10 at 37°C but not at 42°C. In contrast to this, intact Bo2h-F89 phage was infectious for SN2 and SN10 at both temperatures. When transfection experiments were carried out with SN2 competent cells and Bo2h-F89 DNA at 37°C, only a zone of hazy confluent lysis was produced using a lawn of either F89 or SN2. Intact phage Bo2h-F89 at high

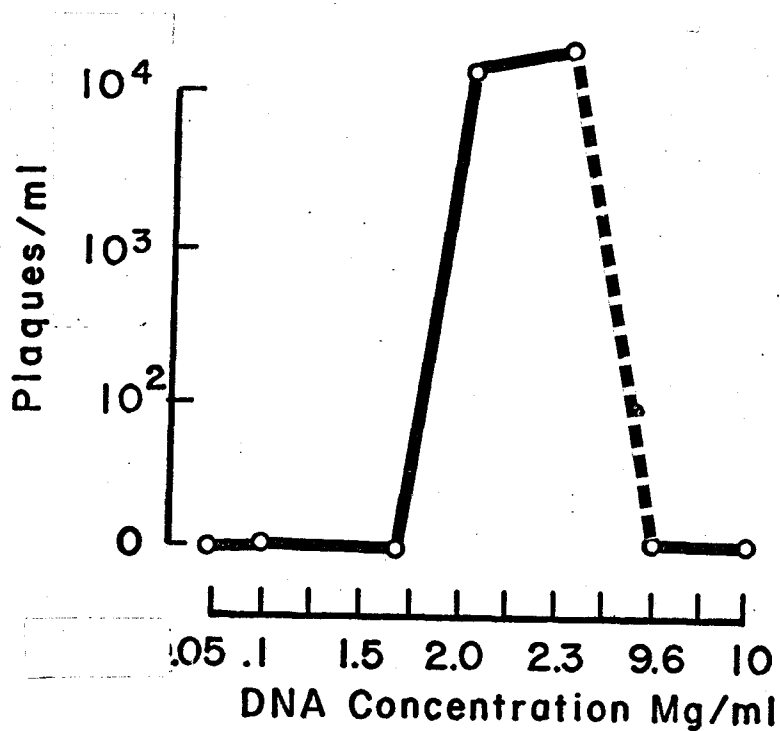


Figure 8. The effect of DNA concentration on transfection.

A competent population of *M. phlei* F89 was cultured for 48 hours with Bo2h·F89 phage DNA at specified concentrations. Reactions were terminated by the addition of 20 μ g/ml DNase.

o—o Infectious centers were assayed for over entire range.

o----o Samples were not taken in this concentration range.

concentrations produced clear confluent lysis (CL +) whether plated on SN2 or F89 indicator strains. Individual plaques could be obtained in each case by serial dilution of the concentrated phage preparation. By contrast the hazy zone of confluent lysis (CL (+)) when serially diluted in nutrient medium did not produce distinct plaques but rather an identical hazy clearing which gradually diminished with successive dilutions. On the other hand, when transfection experiments were carried out at 42°C with Bo2h-F89 using SN2 as the host bacterium, individual plaques could be obtained analogous to those of transfection of M. phlei F89 or M. smegmatis SN10 at 37°C. These results are summarized in Table 5.

Host Strain	37°C Bo2h·F89			42°C Bo2h·F89		
	Intact Phage	DNA	DNA + DNase	Intact Phage	DNA	DNA + DNase
F89	CL+	CL+	0	CL+	0	0
SN10	CL+	CL+	0	CL+	0	0
SN2	CL+	CL(+)	0	CL+	CL+	0

TABLE 5. The effect of temperature on the infectivity of phage Bo2h·F89 and its DNA. Bo2h·F89 phage or DNA extracted from it were reacted with competent M. phlei F89, M. smegmatis SN2 or SN10 cells either at 37°C or at 42°C for 48 hours. Transfection reaction was terminated by the addition of DNase at a concentration of 20 µg/ml.

CL+ : clear lysis, individual plaques obtainable on
dilution

CL(+): hazy lysis, no individual plaques can be recovered

0 : no lysis, no infectious centers

DISCUSSION

These experiments have shown that when M. smegmatis SN2 str-s or M. phlei F89 xyl⁻ are cultured in nutrient medium containing 1 to 5 μ moles/ml of CaCl₂, a competent population of cells appear during the stationary phase. In the presence of biologically active M. phlei SN109 xyl⁺, Bo2.SN109 xyl⁺, M. smegmatis SN2 str-r or SN46 str-r DNA, these cells (depending on the marker used, either xyl⁺ or str-r) seem to be able to incorporate into their genome(s) the respective marker. This was indicated by the fact that F89 recipient cells were able to grow in a minimal medium containing only D-xylose as sole carbon source after being treated with either SN109 xyl⁺ or Bo2.SN109 xyl⁺ DNA. Similarly, SN2 str-r cells were able to grow in a medium containing high concentrations of streptomycin after exposure to either SN46 str-r or SN2 str-r donor DNA. It was further confirmed by our transfection experiments that competence is actually achieved during the stationary phase.

Transfection studies have indicated that strains M. smegmatis SN2 and M. phlei F89 are suitable recipients of biologically active DNA. It was observed that the uptake and subsequent expression of phage DNA was both time and concentration dependent. The interpretation given the data in Figures 5, 6 and 7 was that a low number of cells of a stationary culture remained competent for phage DNA for approximately 2 to 3 hours and then lost competence thereafter. If the cells were allowed to recycle to the stationary phase in the presence of DNA, a larger number of cells in the population became competent and the DNA was taken up and expressed by them. The increased number of infectious centers is believed to have been due to the greater number of competent cells and the longer period of

contact time (48 hours), which permitted reassembled phage particles to go through several cycles of multiplication. Romig (39) was the first to report that greater numbers of infectious centers were obtained in transfection experiments with B. subtilis phage SP3 DNA if the reassembled particles were allowed to go through several cycles of multiplication. In addition, it was observed that the phage DNA preparations were infectious over a narrow range of DNA concentration (Fig. 8). The interpretation of Green (25,26) and Romig (39) may partially explain our results. Romig (39) reported a nonlinear relationship between SP3 bacteriophage DNA and the number of infectious centers in B. subtilis. In those experiments he also found that phage DNA concentrations below 1 $\mu\text{g/ml}$ were incapable of producing infectious centers. As mentioned before in our experiments no infectious centers appeared with an input of less than 2 $\mu\text{g/ml}$ of DNA. As for SP82, Green (25,26) has presented evidence that the nonlinear relationship between the amount of input DNA and the number of reassembled infectious bacteriophages is due to recombination between transfecting phage DNA molecules. He suggested that genetic recombination between incoming phage DNA is necessary for the production of complete phage molecules. It could not be determined why our DNA preparations behaved as they did at concentrations higher than 2.3 $\mu\text{g/ml}$. One possible explanation might be that either the physiological state of the bacteria, the number of competent cells or a "high" DNA concentration favor lysogenization. Evidence which may support the latter hypothesis was recently obtained from transformation experiments by transfection employing the xylose marker.

In these experiments, DNA extracted from phage Bo2 SN109 xyl^+ was capable of transferring the xylose marker to a competent culture of

recipient M. phlei F89 xyl⁻ (Exp. 3, Table 2). When the transformants were subcultured in HIB and incubated for 48-72 hours at 37°C, infectious phage(s) were obtained thus indicating that transformation of the xyl⁻ character was accompanied by transfection. These results suggest that lysogeny may play a role in transfection and/or transformation by transfection. This single genetic marker (D-xylose utilization) could play a significant role in elucidating the mechanism(s) controlling the uptake and subsequent expression of phage DNA molecules. I view this as a significant contribution to future genetic studies in Mycobacterium.

Several other interesting aspects of these transfection studies may relate to transformation in Mycobacterium. For example, it was found that M. phlei strain F89 was competent for the uptake of phage Bo2h DNA molecules during the stationary phase of growth at 37°C but plaques were not formed when the temperature was raised to 42°C. By contrast, the SN2 host developed competence for the uptake of phage Bo2h·F89 DNA during the stationary phase at 42°C and while competent for phage DNA uptake at 37°C, no individual plaques were produced at this temperature (see Table 5). It would be very significant to find the cause(s) for these temperature effects on transfection. It is tempting to speculate that DNA penetration is dependent on a highly specific, host-controlled mechanism and that although capable of penetration through the host cell wall the DNA is either partially degraded or blocked in its replication once inside the host bacterium. This might be what happens at 37°C in our transfection experiments with SN2 host bacterium.

There is some evidence that in B. subtilis the initial stages of transformation and transfection are identical (15). This is also true in

Hemophilus influenzae (29). It has been shown that bacterial strains which restrict reassembly of phage from its DNA restrict similarly transformation by bacterial DNA (5). The data indicate that SN2 is partially restrictive to phage Bo2h DNA at 37°C but that this restriction is overcome at 42°C. Concerning future transformation experiments, an attempt will be made to show that transformation of auxotrophic strains (recently made available to our laboratory by Dr. K.T. Holland) to prototrophy is similarly affected by differences in temperature. The effect of temperature on host-controlled restriction has been studied by Schell and Glover (41) in E. coli. They demonstrated that in E. coli K (P1), "phage λ .c (i.e. phage λ grown in E. coli c) is normally restricted so that the efficiency of plating of phage λ .c on K (P1) compared to C is about 10^{-7} . When the E. coli K (P1) population is heated before infection with phage λ , this restriction can be decreased as much as a million-fold. Heated K (P1) bacteria recovered their ability to restrict phage λ .c following resumption of growth at 37°C."

The reported transfection studies have indicated that M. phlei restricts phage Bo2h DNA but not intact phage at 42°C (Table 5). Similar results were obtained in experiments when transformation was performed at 37°C in contrast to those at 42°C using the xylose marker. The data in Table 2, Exps. 2 and 3, indicate that M. phlei F89 appears to restrict both phage Bo2-SN109 xyl^{+} DNA and SN109 xyl^{+} bacterial DNA at 42°C. The effect of temperature upon genetic transfer of the xylose marker might be used to great advantage in clarifying the mechanism(s) for what seems to be a temperature-dependent restriction phenomenon in this recipient. Experiments have been initiated which aim at clarifying the effects of temperature, the kinetics of DNA uptake and the mechanism(s) of phage DNA infection in this host bacterium.

In conclusion, the experiments reported on the transformation of the xylose marker are viewed as a significant first in the genus Mycobacterium and this apparent success may be attributed primarily to the gentle procedure used for the isolation of bacterial DNA; the failure of other investigators to demonstrate transformation in this genus apparently was due to the extremely "harsh" methods employed to isolate donor bacterial DNA.

SUMMARY

Evidence has been presented in this thesis for a transformation system in Mycobacterium. A significant level of transfer of the xylose marker was observed in a system using M. phlei SN109 xyl^+ DNA as donor. A competent stationary culture (48 hours) of M. phlei F89 xyl^- was used as recipient. The data indicate that transformation occurred for the first time in the genus Mycobacterium. By contrast, it is merely suggested that streptomycin resistance is transferable to stationary phase SN2 str-s cells by either SN2 str-r or SN46 str-r DNA preparations. The efficiency of transformation to str-r is low but reproducible. The role of bacterial DNA in either system was substantiated by loss of transforming activity when deoxyribonuclease was added or when recipient cells were cultured without DNA. A model transfection system was used to study competence of M. phlei strain F89 and M. smegmatis SN2. Phage Bo2h DNA could transfect both strains during the stationary phase; the reaction was inhibited by the addition of DNase. Furthermore, the transfection process was affected by alteration of the temperature.

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APPROVAL SHEET

This thesis submitted by Louis A. DeSalle, III has been read and approved by three members of the Faculty of the Graduate School.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

This thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 24, 1971
Date

Steph E. Juhar
Signature of Advisor